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Stable carbon isotope fractionation by marine phytoplankton in response to daylength, growth rate, and CO_2 availability

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ABSTRACT: Stable carbon isotope fractionation (ε_p) of 7 marine phytoplankton species grown in different irradiance cycles was measured under nutrient-replete conditions at a high light intensity in batch cultures. Compared to experiments under continuous light, all species exhibited a significantly higher instantaneous growth rate (μ_i), defined as the rate of carbon fixation during the photoperiod, when cultivated at 12:12 h, 16:8 h, or 18:6 h light:dark (L/D) cycles. Isotopic fractionation by the diatoms *Skeletonema costatum*, *Asterionella glacialis*, *Thalassiosira punctigera*, and *Coscinodiscus wailesii* (Group I) was 4 to 6‰ lower in a 16:8 h L/D cycle than under continuous light, which we attribute to differences in μ_i. In contrast, ε_p in *Phaeodactylum tricornutum*, *Thalassiosira weissflogii*, and in the dinoflagellate *Scrippsiella trochoidea* (Group II) was largely insensitive to daylength-related differences in instantaneous growth rate. Since other studies have reported growth-rate dependent fractionation under N-limited conditions in *P. tricornutum*, μ_i-related effects on fractionation apparently depend on the factor controlling growth rate. We suggest that a general relationship between ε_p and $\mu_i/[CO_{2,aq}]$ may not exist. For 1 species of each group we tested the effect of variable CO_2 concentration, $[CO_{2,aq}]$, on isotopic fractionation. A decrease in $[CO_{2,aq}]$ from ca 26 to 3 µmol kg^{-1} caused a decrease in ε_p by less than 3‰. This indicates that variation in μ_i in response to changes in daylength has a similar or even greater effect on isotopic fractionation than $[CO_{2,aq}]$ in some of the species tested. In both groups ε_p tended to be higher in smaller species at comparable growth rates. In 24 and 48 h time series the algal cells became progressively enriched in ^{13}C during the day and the first hours of the dark period, followed by ^{13}C depletion in the 2 h before beginning of the following light period. The daily amplitude of the algal isotopic composition ($\delta^{13}C$), however, was ≤1.5‰, which demonstrates that diurnal variation in $\delta^{13}C$ is relatively small.

KEY WORDS: Isotope fractionation · ^{13}C discrimination · Growth rate · CO_2 · Paleobarometer · Daylength · Diurnal variation · Diatoms · Phytoplankton

INTRODUCTION

The stable carbon isotopic composition ($\delta^{13}C$) of marine phytoplankton depends on both the environmental conditions and the physiology of algal cells. It is determined by (1) the isotopic composition of the inorganic carbon source, (2) enzymatic isotope fractionation during inorganic carbon fixation, and (3) further modification of $\delta^{13}C$ of assimilated carbon compounds during carboxylation/decarboxylation reactions in respiration and secondary biosynthetic pathways. Knowledge of the relative contribution of each of these factors to natural variability in $\delta^{13}C$ is required to interpret distributions of stable isotopes in marine plankton.

A systematic depletion in ^{13}C relative to ^{12}C in both plankton and sediment organic carbon from low to higher latitudes was first reported by Sackett et al. (1965) and has been confirmed in subsequent studies (e.g. Sackett et al. 1974, Rau et al. 1982, 1989). Covariance of $\delta^{13}C$ of suspended particulate organic matter with concentrations of dissolved molecular carbon dioxide ($[CO_{2,aq}]$) in ocean surface waters led to the suggestion that ambient CO_2 concentrations might be

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the major determinant of $\delta^{13}C$ in marine phytoplankton (e.g. Deuser et al. 1968, Rau et al. 1989, 1992). If $[CO_{2,aq}]$ controls $\delta^{13}C$ values in primary produced organic matter and if little modification occurs during sedimentation, variation in the isotopic composition of organic compounds in the sedimentary record could serve as a 'paleobarometer' to reconstruct CO_2 concentrations in sea surface water over geologic time scales (e.g. Jasper & Hayes 1990, Freeman & Hayes 1992, Jasper et al. 1994, Rau 1994).

Several lines of evidence, however, indicate that factors other than CO_2 concentration may also account for significant variability of $\delta^{13}C$ in plankton. In the field, differences between species and differences in growth rates (μ), which further depend on environmental factors such as temperature, light intensity and nutrient supply, may be similar in importance to $[CO_{2,aq}]$ in affecting the isotopic composition of marine phytoplankton (e.g. Francois et al. 1993, Goericke & Fry 1994, Pancost et al. 1997, Kukert & Riebesell 1998).

Several models of photosynthetic ^{13}C fractionation in marine microalgae based on diffusive molecular CO_2 uptake demonstrate that species-specific parameters can play a significant role in accounting for observed variations of $\delta^{13}C$ in oceanic plankton (e.g. Francois et al. 1993, Goericke et al. 1994, Laws et al. 1995, 1997, Rau et al. 1996, 1997, Popp et al. 1998). Such parameters include CO_2 permeability of the cell membrane, enzymatic fractionation, cellular carbon content, and the cell surface area to volume ratio.

Laboratory experiments with the diatoms *Phaeodactylum tricornutum* (Laws et al. 1995), *Porosira glacialis* (Popp et al. 1998), and the coccolithophorid *Emiliania huxleyi* (Bidigare et al. 1997) in nitrate-limited continuous cultures reveal a highly significant, linear inverse relationship between isotopic fractionation (ε_p) and the ratio of growth rate and $[CO_{2,aq}]$ at CO_2 concentrations above ca 9 µmol kg^{-1}. Differences in the slope of the regression lines can be accounted for by differences in the surface area and carbon content of the cells (Popp et al. 1998). Such correlation is in accordance with the theory of diffusive uptake of CO_2 during algal growth, but might be equally well explained by an active transport mechanism of either CO_2 or HCO_3^- into the cell (Keller & Morel 1999). Regardless of the mechanism of inorganic carbon uptake, this relationship may be applicable in the field to estimate growth rates from measurements of $\delta^{13}C$ and $[CO_{2,aq}]$ if the linear dependence of ε_p on $\mu/[CO_{2,aq}]$ proves to be a general phenomenon.

In the above-mentioned experiments, growth rate and $[CO_{2,aq}]$ were varied simultaneously so that it is not possible to distinguish between growth rate effects, CO_2 effects and their relative contribution to variability in ε_p. Similarly, $[CO_{2,aq}]$ was not constant in experiments by Fry & Wainright (1991), who reported a linear inverse relationship between ε_p and μ. Interpretation of their data is further complicated by differences in species composition in mixed cultures dominated by either fast-growing diatoms or slow-growing dinoflagellates.

It is also important to keep in mind that isotopic fractionation reflects carbon assimilation during the photoperiod (Laws et al. 1995). Thus, growth rate estimates should account for light:dark (L/D) cycles by calculating instantaneous growth rates (μ_i) from 24 h average growth rates (μ_{L+D}). Instantaneous rates of carbon assimilation in the light may differ by a factor of up to 2 between algae grown at different L/D cycles when their 24 h average growth rate is the same. Under natural conditions, large latitudinal and seasonal differences in daylength exist. Considering an algal cell growing at 12 h of daylight in equatorial surface waters at the same 24 h average rate as under near-continuous light conditions during summer at high latitudes, it needs to assimilate CO_2 at approximately twice the rate.

It is the main goal of our study to quantify the effect of variable growth rate on stable carbon isotopic fractionation at constant $[CO_{2,aq}]$ and compare μ-dependent changes in ε_p between several species of marine phytoplankton of different sizes under controlled laboratory conditions. For this purpose we incubated 6 diatom and 1 dinoflagellate species in diluted batch cultures at 15°C under nutrient replete conditions at a high light intensity. Concentrations of $CO_{2,aq}$ were adjusted to 25–37 µmol kg^{-1}, which is approximately 2 to 3 times the concentration in natural seawater at that temperature. Variation in growth rates was achieved by acclimating the algae to different L/D cycles. This approach makes use of the ability of many marine phytoplankton species to keep their 24 h average growth rate relatively constant, independent of duration of the photoperiod (Brand & Guillard 1981). Consequently, μ_i during the photoperiod increases when algae are transferred from continuous light to a L/D cycle.

In additional experiments, the effect of variable $[CO_{2,aq}]$ on ε_p at constant μ_i was measured in 2 of the diatom species which exhibited little variation in growth rate between CO_2 concentrations of ca 3 and 25 µmol kg^{-1}. Furthermore, diurnal variation in $\delta^{13}C$ of these diatoms was monitored in 2 h intervals to determine the potential impact of metabolic processes such as respiration and biosynthesis in the dark on the isopic composition of the diatom cultures. Differences in the response of ε_p to variation in μ_i and $[CO_{2,aq}]$ between individual species in our study are discussed with respect to possible mechanisms and pathways of inorganic C acquisition in marine phytoplankton.

MATERIALS AND METHODS

Experiments. Dilute batch cultures of the marine diatom species *Skeletonema costatum*, *Thalassiosira weissflogii*, *Thalassiosira punctigera*, *Phaeodactylum tricornutum*, *Asterionella glacialis*, *Coscinodiscus wailesii*, and of the marine dinoflagellate *Scrippsiella trochoidea* were grown in Rumed 1200 light-thermostats at a temperature of 15°C and an incident photon flux density of 150 µmol photons m^{-2} s^{-1}. The growth medium consisted of 0.2 µm filtered, nutrient-enriched natural seawater with nitrate, silicate, and phosphate concentrations of 100, 100, and 6.25 µmol kg^{-1}, respectively. Trace metals, EDTA, and vitamins were added according to *f*/2-enrichment (Guillard & Ryther 1962). Depending on the batch of seawater, salinity varied between 30.5 and 31.5 psu. Stock cultures were pre-adapted to experimental conditions of the respective treatments for at least 9 cell divisions.

All experiments were performed in 2.4 l borosilicate glass bottles, sealed with PBT-lined screw caps. Initial tests indicated that, after inoculation at low cell concentrations, photosynthetic fixation of CO_2 in the closed experimental system caused an increase in pH by ≤0.05 when cells where harvested at concentrations equivalent to ca 50 µmol kg^{-1} particulate organic carbon (POC). To minimize pH drift and associated shifts in carbon speciation, cells in all treatments were typically harvested at POC concentrations of 20 to 30 µmol kg^{-1}. Another reason to avoid dense cultures is a concomitant change in $\delta^{13}C$ of dissolved inorganic carbon (DIC) during discrimination against ^{13}C in photosynthesis. In our dilute cultures, changes in the isotopic composition of DIC never exceeded 0.4‰ during the experiments.

Prior to the experiments, growth rates were determined for adapted cells and cellular carbon content was estimated from cell volume according to Strathmann (1967). This information together with measurements of cell concentrations of the pre-cultures was used to calculate the appropriate amount of inoculum for each bottle to permit ca 9 cell divisions before the target concentration of <50 µmol kg^{-1} POC was reached and the experiment was terminated. In all of these experiments, cells were harvested at the same time of the day, corresponding to the end of the 16 h photoperiod. Microscopical analyses of subsamples indicated that bacterial biomass never exceeded 1% of algal biomass in the cultures and its contribution to total POC was therefore considered negligible.

Skeletonema costatum and *Thalassiosira weissflogii* were used to monitor diurnal variation in $\delta^{13}C$ over a 24 or a 48 h time period. The 12 or 24 bottles required for each treatment of a time series were inoculated with pre-adapted diatom culture simultaneously in such a way that the appropriate concentration of POC of <50 µmol kg^{-1} was reached at the time of sampling. In all treatments, samples for the parameters of interest were then taken at 2 h intervals. For that purpose, incubation bottles were harvested completely and subsamples were used for chemical and microscopical analysis. In treatments including a dark phase, the first sample was taken at the beginning of the light period. Variable concentrations of $CO_{2,aq}$, ranging from 2.6 to 25.8 µmol kg^{-1}, were obtained by adjusting pH with 1 N HCl or 1 N NaOH to values between 7.97 and 8.87 (Table 1). *S. costatum* was grown at L/D cycles of 12:12, 16:8, and 24:0 h. *T. weissflogii* was grown at L/D cycles of 18:6 and 24:0 h. All other species were incubated at high $[CO_{2,aq}]$ of 30.1 to 37.7 µmol kg^{-1} at both 16:8 and 24:0 h L/D cycles (Table 1).

Carbonate system. Experimental CO_2 concentrations (Table 1) were adjusted by changing the relative proportions of the inorganic carbon species $CO_{2,aq}$, HCO_3^-, and CO_3^{2-} through addition of HCl or NaOH. This causes pH to vary together with $[CO_{2,aq}]$ and alkalinity at constant DIC concentration. Lower pH favors equilibrium reactions between CO_2 and HCO_3^- in the direction of CO_2. Minor variation in DIC between treatments listed in Table 1 resulted from differences between seawater batches and from some CO_2 gas exchange after manipulating the carbonate system before the bottles were closed. It should be pointed out that the approach chosen in other studies (e.g. Laws et al. 1995, Johnston 1996, Bidigare et al. 1997), in which $[CO_{2,aq}]$ is manipulated by aeration at different CO_2-levels, results in similar changes in pH upon $[CO_{2,aq}]$ variation as in our treatments. In an aerated system, however, alkalinity remains constant at variable DIC.

$[CO_{2,aq}]$ was calculated from DIC concentrations, total alkalinity (tAlk), temperature, salinity, and concentrations of phosphate and silicate assuming dissociation constants according to Mehrbach et al. (1973). DIC was measured in duplicate by coulometric titration, tAlk was determined in duplicate by Gran-titration, and pH was both calculated from DIC and tAlk and measured potentiometrically. For a detailed description of the methods see Burkhardt & Riebesell (1997).

Stable carbon isotope fractionation. Samples were filtered using precombusted (500°C, 12 h) Whatman GF/C glass fiber filters. All filters were acidified with 0.1 N HCl prior to measurements and dried for 12 h at 60°C. Analysis of isotopic composition of particulate organic carbon ($\delta^{13}C_{POC}$) was performed in duplicate on a mass spectrometer (ANCA-SL 20-20 Europa Scientific). Isotopic composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) was analyzed on unfiltered subsamples, preserved with $HgCl_2$ (3.5% w/v final concentration), with a Finnegan MAT Delta-S mass spectrometer after acidification with 100% H_3PO_4 at 25°C in a vacuum extraction line, as described by Mackensen et al. (1996). The

Table 1. Experimental conditions, isotope measurements, and growth rate estimates during dilute batch culture incubations of 7 marine phytoplankton species. Results for *Skeletonema costatum* and *Thalassiosira weissflogii* are mean values of 24 or 48 h time series (see text for definition of variables)

Species	L/D (h:h)	DIC (mmol kg^{-1})	tAlk (meq kg^{-1})	pH	[$CO_{2,aq}$] (µmol kg^{-1})	$\delta^{13}C_{CO_2}$ (‰)	$\delta^{13}C_{POC}$ (‰)	ε_p (‰)	μ_{L+D} (d^{-1})	μ_i (d^{-1})
Group I										
Skeletonema costatum	12:12	2.11	2.31	8.14	17.3	−10.7	−17.6	7.0	1.6	3.8
	16:8	2.10	2.90	8.87	2.6	−10.7	−16.8	6.2	2.0	3.3
	16:8	2.11	2.31	8.14	17.3	−10.7	−18.0	7.4	2.1	3.4
	16:8	2.10	2.23	7.98	25.5	−10.7	−18.5	8.0	2.1	3.4
	24:0	2.10	2.90	8.87	2.6	−10.7	−22.9	12.4	1.7	1.7
	24:0	2.10	2.23	7.98	25.5	−10.7	−24.5	14.1	1.9	1.9
Asterionella glacialis	16:8	2.14	2.24	7.86	33.8	−10.4	−18.3	8.1	1.8	2.9
	24:0	2.14	2.24	7.86	33.8	−10.4	−22.2	12.1	1.7	1.7
Thalassiosira punctigera	16:8	2.06	2.19	7.90	30.1	−10.0	−17.0	7.1	0.8	1.3
	24:0	2.06	2.19	7.90	30.1	−10.0	−21.9	12.1	0.9	0.9
Coscinodiscus wailesii	16:8	2.08	2.15	7.83	35.7	−10.2	−11.8	1.6	0.6	0.9
	24:0	2.08	2.15	7.83	35.7	−10.2	−17.7	7.6	0.3	0.3
Group II										
Phaeodactylum tricornutum	16:8	2.05	2.10	7.80	37.7	−10.3	−25.9	16.0	1.4	2.3
	24:0	2.05	2.10	7.80	37.7	−10.3	−26.6	16.7	1.6	1.6
Thalassiosira weissflogii	18:6	2.09	2.76	8.75	3.5	−10.7	−22.5	12.0	1.5	2.1
	18:6	2.07	2.19	7.97	25.8	−10.7	−25.0	14.6	1.5	2.1
	24:0	2.09	2.76	8.75	3.5	−10.7	−22.5	12.1	1.6	1.6
	24:0	2.07	2.19	7.97	25.8	−10.7	−24.6	14.3	1.6	1.6
Scrippsiella trochoidea	16:8	2.08	2.15	7.83	35.7	−10.2	−19.8	9.7	0.6	0.9
	24:0	2.08	2.15	7.83	35.7	−10.2	−18.3	8.2	0.6	0.6

carbon isotopic composition is reported in δ-notation relative to PeeDee belemnite as a standard:

$$\delta^{13}C = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1\right] 1000 \quad (1)$$

The isotopic composition of dissolved molecular CO_2 ($\delta^{13}C_{CO_2}$) was calculated from $\delta^{13}C_{DIC}$ following the equation provided by Rau et al. (1996) based on Mook et al. (1974):

$$\delta^{13}C_{CO_2} = \delta^{13}C_{DIC} + 23.644 - \frac{9701.5}{T_k} \quad (2)$$

where T_k is the absolute temperature in kelvin.

The isotopic fractionation associated with photosynthetic CO_2 fixation, ε_p, was calculated relative to the isotopic composition of CO_2 in the bulk medium according to Freeman & Hayes (1992):

$$\varepsilon_p = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \delta^{13}C_{POC}/1000} \quad (3)$$

Growth rates. Determination of growth rates was based on either cell counts or POC measurements. The average 24 h growth rate was calculated according to

$$\mu_{L+D} = \frac{\ln X_i - \ln X_{i-1}}{\Delta t} \quad (4)$$

where X_{i-1} and X_i are concentrations of algal cells or POC at the beginning and at the end of the time interval Δt. Duplicate 20 ml subsamples, preserved with Lugol's iodine (Edler 1979), where used for cell counts. Cell concentrations of *Phaeodactylum tricornutum* were determined with a Coulter 'Multisizer'. Cell concentrations of all other species were obtained from enumeration under the inverted microscope in duplicate on each subsample. Cell numbers in control bottles were determined from cell enumerations daily (slowly growing cells) or twice a day (rapidly growing cells) for at least 3 subsequent days parallel to each experimental run. From the experimental vessels, no subsamples were taken during incubation to avoid disturbance of the closed system, but cell concentrations were determined before and after incubation and calculated growth rates were compared to the control bottles.

Growth rates listed in Table 1 are based on concentrations of POC at the beginning ($[POC]_0$) and the end ($[POC]_1$) of incubation. $[POC]_1$ was measured directly in the analysis of $\delta^{13}C_{POC}$ as described above. $[POC]_0$ was calculated from the initial cell concentration and cellular carbon content. Growth rates determined from POC rather than cell concentrations permit the intro-

duction of a term for dark carbon loss to account for differences in daylength between treatments. μ_i, which represents the rate of photosynthetic carbon assimilation during the light period, was calculated according to

$$\mu_i = \frac{(L+D)\,\mu_{L+D}}{L - D\,r} \quad (5)$$

L and D are duration of the light and the dark period, respectively. The rate of carbon loss in the dark relative to the rate of carbon assimilation during the preceding photoperiod was assumed to equal 15% (Laws & Bannister 1980), in which case r equals 0.15 in Eq. (5).

RESULTS

Growth rates based on cell counts at the beginning and the end of incubation deviated by ≤ 0.1 d^{-1} from rates obtained from successive cell counts in pre-cultures. This indicates that no extended lag phase, which would affect calculation of growth rates from 2-point measurements, preceded exponential growth during the experiments. It becomes obvious from Table 1 that all species except *Coscinodiscus wailesii* exhibited only minor differences in μ_{L+D} between growth under continuous light or at a L/D cycle, i.e. cells were able to compensate for up to 8 h of darkness by enhanced rates of carbon assimilation during the photoperiod. *Skeletonema costatum* even grew at a somewhat higher rate in the 16:8 h L/D cycle than in continuous light. Only an extension of the dark period to 12 h resulted in a decrease in growth rate at comparable $[CO_{2,aq}]$. In *S. costatum* and *Thalassiosira weissflogii*, a decrease in $[CO_{2,aq}]$ from ca 25 to 3 µmol kg^{-1} had little effect on μ_{L+D}. As a result of relatively constant μ_{L+D} within each species, μ_i at a 16:8 or 18:6 h L/D cycle exceeded μ_i in continuous light by a factor of 1.6 or 1.4 when it is assumed that carbon loss in darkness accounts for 15% of the carbon assimilated during the preceding photoperiod.

We observed only small differences in $\delta^{13}C_{CO_2}$ (−10.0 to −10.7‰) between different batches of seawater used in our experiments, and no significant drift in $\delta^{13}C$ of the carbon source occurred during the experiments listed in Table 1. The 7 phytoplankton species investigated can be divided into 2 groups according to their response in ε_p to variable μ_i at constant $[CO_{2,aq}]$. In Group I (Fig. 1a), ε_p during growth under continuous light was up to 6‰ higher than in a 16:8 h L/D cycle under otherwise identical conditions. In contrast, the 3 species of Group II (Fig. 1b) showed no response in ε_p to daylength or even somewhat higher ε_p values during growth in a L/D cycle. In both Fig. 1a & b, the species are listed in the order of increasing cell size from the left to the right. As a general trend, isotopic fractionation decreases within each group as cell size increases.

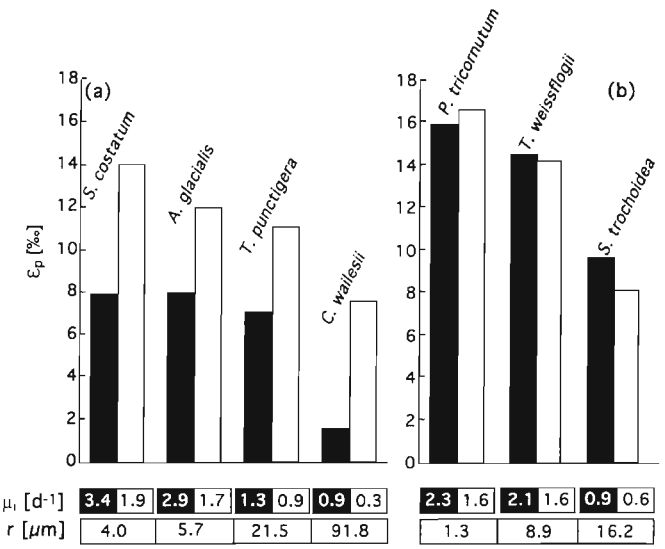

Fig. 1 Stable carbon isotope fractionation (ε_p) in continuous light (open bars) and in a 16:8 h light:dark cycle (18:6 h in *Thalassiosira weissflogii*, solid bars) at $[CO_{2,aq}] > 25$ µmol kg^{-1} (see Table 1). Within each group, cell size of the species increases from left to right. Instantaneous growth rate (μ_i) and surface-equivalent spherical cell radius (r) are shown in the lower panels. Results for *Skeletonema costatum* and *T. weissflogii* are mean values of 24 h time series. See Table 1 for full species' binomials; (a) Group I and (b) Group II

In the time-series experiments with *Skeletonema costatum* and *Thalassiosira weissflogii*, the effect of μ_i on ε_p at constant $[CO_{2,aq}]$ can be compared to the effect of variable $[CO_{2,aq}]$ on ε_p at constant μ_i. Fig. 2 summarizes the results for *S. costatum* (Group I) grown at 4 combinations of $[CO_{2,aq}]$ and L/D cycles, measured in 2 h intervals over a 24 h period. According to our definition of isotopic fractionation (Eq. 3), ε_p refers to differences in the isotopic composition between carbon source and product due to photosynthetic carbon fixation. Thus it cannot be used to characterize processes occurring in darkness. Therefore, we show $\delta^{13}C_{POC}$ instead of ε_p values to describe diurnal trends in Fig. 2. Differences in $\delta^{13}C$, however, are directly related to differences in ε_p because the isotopic composition of CO_2 was identical in these experiments ($\delta^{13}C_{CO_2}$ = −10.7‰). For comparison of isotopic fractionation, we calculated ε_p of the respective treatment from $\delta^{13}C_{POC}$ averaged over a 24 h period (Table 1). In *S. costatum*, we observed a marked effect of μ_i on ε_p induced by the different L/D cycles. During growth under continuous light, fractionation was, on average, 6‰ higher than in the 16:8 h L/D cycle, corresponding to a decrease in μ_i by ca 1.5 d^{-1}. Since differences in μ_i between high and low $[CO_{2,aq}]$ were ≤ 0.2 d^{-1}, it becomes obvious that changes in the carbonate system affected isotopic fractionation in *S. costatum* independent of growth rate. At

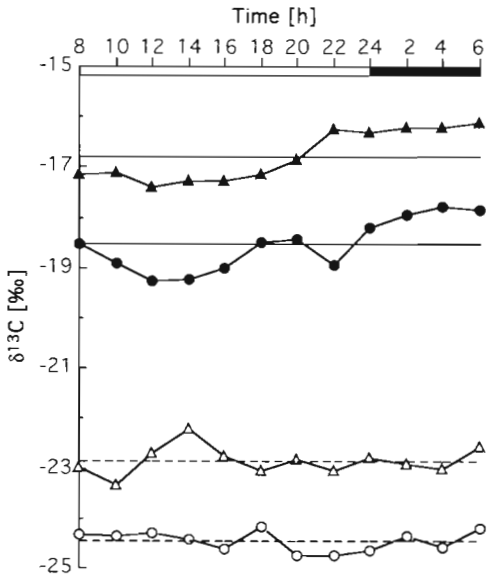

Fig. 2. *Skeletonema costatum*. Diurnal variation in the isotopic composition of POC ($\delta^{13}C_{POC}$) over a 24 h period at L/D cycles of 16:8 h (closed symbols) versus 24:0 h (open symbols) and $[CO_{2,aq}]$ = 2.6 µmol kg^{-1} (triangles) versus 25.5 µmol kg^{-1} (circles). The solid bar indicates the dark period. Mean values of all samples are shown as a reference for each treatment (dashed lines for L/D = 24:0 h)

a given daylength, ε_p was, on average, 1.7 to 1.8‰ higher at 25.5 µmol kg^{-1} $[CO_{2,aq}]$ than at 2.6 µmol kg^{-1} $[CO_{2,aq}]$ (Table 1). Thus the effect of variable CO_2 on ε_p appeared to be small compared to the growth rate effect imposed by an 8 h difference in daylength. The offset in ε_p between different L/D cycles was of the same magnitude at both CO_2 levels and persisted throughout a 24 h period.

Thalassiosira weissflogii represents a species of Group II showing no response in ε_p to a daylength-related difference in μ_i of 0.5 d^{-1} (Table 1, Fig. 3). Although this variation in μ_i is small compared to *Skeletonema costatum* as a result of a shorter dark period in the 18:6 h L/D cycle (multiplying μ_{L+D} with 1.4 instead of 1.6) and lower absolute values of μ_{L+D}, a noticeable difference in ε_p between continuous light and an 18:6 h L/D cycle would still be expected if *T. weissflogii* behaved in a similar way as *S. costatum*. The effect of CO_2 concentration (3.5 vs 25.8 µmol kg^{-1}) on isotopic fractionation was slightly greater in *T. weissflogii* than in *S. costatum* with an average increase in ε_p by 2.6‰ (18:6 h) or 2.2‰ (24:0 h) from low to high $[CO_{2,aq}]$.

In both species, $\delta^{13}C_{POC}$ tended to gradually increase during a 24 h period when grown in 16:8 or 18:6 h L/D cycles (Figs. 2 & 3). No such trend was observed during growth under continuous light. The mean difference in $\delta^{13}C_{POC}$ between duplicate measurements was 0.2‰, which is approximately equal to the precision of the Europa Scientific mass spectrometer used for $\delta^{13}C_{POC}$ measurements. Therefore, we conclude that the observed amplitude of ca 1.5‰ in diurnal variation of $\delta^{13}C_{POC}$ was significant, since it exhibited a systematic trend rather than random variation and could be reproduced in a 48 h experiment with *Skeletonema costatum* (Fig. 4). In both species we found $\delta^{13}C_{POC}$ values averaged over the light period to be significantly lower (Student's *t*-test, $p < 0.01$) than in darkness. In contrast, $\delta^{13}C_{POC}$ values averaged over the first 16 or 18 h of a 24 h sampling period were not different from mean $\delta^{13}C_{POC}$ of the remaining samples in experiments under continuous light. Parallel trends at different CO_2 concentrations provide additional evidence for diurnal changes in $\delta^{13}C_{POC}$. To further evaluate this diurnal pattern, we monitored $\delta^{13}C_{POC}$ of *S. costatum* in an additional experiment over 48 h in a 16:8 h L/D cycle. Fig. 4 indicates that minimum $\delta^{13}C$ values again were obtained during the first hours of the light period and that the observed pattern was repeated during the following day.

Since the results for the 24 h time-series experiments were obtained independent of each other from 12 individual bottles per treatment, we can use the data to evaluate the replicability of $\delta^{13}C_{POC}$ measurements in our study. The narrow range of ≤1‰ variation in $\delta^{13}C_{POC}$ under continuous light (Figs. 2 & 3) indicates that our experimental approach, in which cells were harvested at low cell densities during exponential

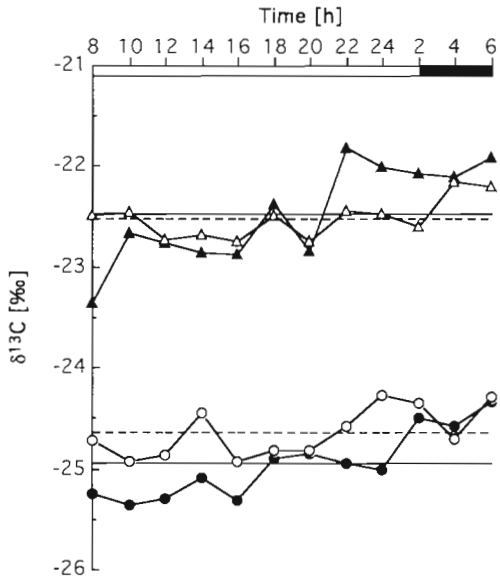

Fig. 3. *Thalassiosira weissflogii*. Diurnal variation in $\delta^{13}C_{POC}$ over a 24 h period at L/D cycles of 18:6 h (closed symbols) versus 24:0 h (open symbols) and $[CO_{2,aq}]$ = 3.5 µmol kg^{-1} (triangles) versus 25.8 µmol kg^{-1} (circles). Mean values of all samples are shown as a reference for each treatment (dashed lines for L/D = 24:0 h)

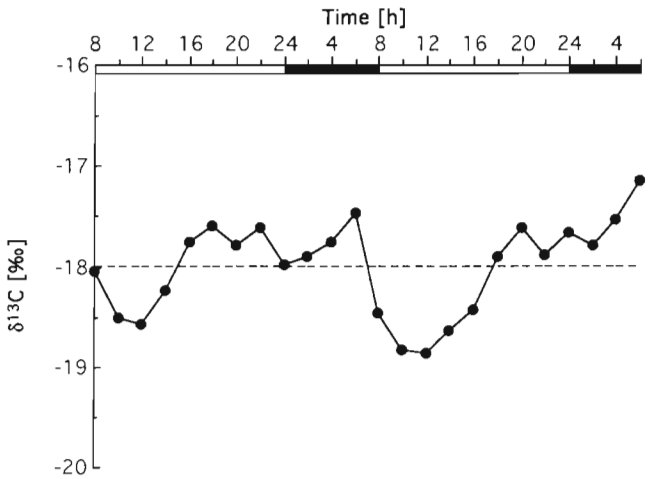

Fig. 4. *Skeletonema costatum*. Diurnal variation in $\delta^{13}C_{POC}$ over a 48 h period at a L/D cycle of 16:8 h and $[CO_{2,aq}]$ = 17.3 µmol kg^{-1} (circles). Mean values of all samples are shown as a reference (dashed line)

growth, is well suited to derive reliable and highly reproducible fractionation values. Considering the accuracy of ca 0.2‰ in $\delta^{13}C_{DIC}$ measurements, we conclude that the 4 to 6‰ difference in isotopic fractionation observed within each species of Group I (Fig. 1) is not caused by random intraspecific variability but is clearly related to differences in irradiance cycles. Further evidence for daylength-dependent fractionation is provided by recent experiments of Burkhardt et al. (in press), who found a similar offset between ε_p values, when *Asterionella glacialis*, *Thalassiosira punctigera*, and *Coscinodiscus wailesii* were incubated at 16:8 and 24:0 h L/D cycles at additional CO_2 concentrations. As in this study, isotopic fractionation of *Phaeodactylum tricornutum* and *Scrippsiella trochoidea* was insensitive to differences in daylength at every experimental $[CO_{2,aq}]$

DISCUSSION

Effect of daylength

In contrast to other studies, in which changes in growth rate were achieved by nutrient limitation, variable light intensity, or differences in species composition of mixed phytoplankton assemblages (e.g. Fry & Wainright 1991, Takahashi et al. 1991, Laws et al. 1995), the wide range of μ_i in the present study is a result of the ability of most species tested here to adjust their rate of carbon fixation to duration of the photoperiod. Such response in μ_i to daily light dose appears to be a common phenomenon among microalgae (Brand & Guillard 1981).

Considering light effects on isotopic fractionation in phytoplankton, it makes a fundamental difference whether daily light dose is reduced by a longer dark period or by lower light intensities. During growth under high light conditions in a L/D cycle, organic carbon is produced at a high rate with sufficient light energy available for metabolic reactions. In contrast, an algal cell under continuous low light conditions may receive the same daily light dose under permanent energy limitation. Limited light supply may be of particular importance if an energy-consuming active transport step is involved in inorganic carbon acquisition, as has been suggested for several phytoplankton species (e.g. Badger & Price 1992). As a result of physiological adaptation to low irradiance levels, marine algae are able to increase photosynthetic quantum yield which is not a parameter that correlates with daylength (Sakshaug & Andresen 1986, Sakshaug et al. 1989). In contrast, adaptation of growth to extended dark periods requires higher photosynthetic rates during the light phase. Experiments by Mortain-Bertrand et al. (1987a,b, 1988a,b) indicate that the photoperiod might directly influence cellular activity of RUBISCO and thus the efficiency of algal cells to use the available inorganic carbon for assimilation into organic compounds.

Few experimental studies have investigated the effect of daylength on isotopic fractionation. In dense batch cultures, Thompson & Calvert (1994) examined the combined effect of photon flux density and daylength on carbon isotope fractionation by the marine diatom *Thalassiosira pseudonana*. However, interpretation of their results is complicated because effects of light intensity and daylength were not treated separately, and observed trends were not consistent over the respective ranges. Leboulanger et al. (1995) compared the isotopic composition of 3 marine microalgae (*Skeletonema costatum*, *Isochrysis galbana*, *Amphidinium operculatum*), grown in batch culture under light-limitation with different L/D cycles. All species were enriched in ^{13}C by 1.5 to 3.2‰ when grown in a 12:12 h L/D cycle compared to continuous light. Assuming that $\delta^{13}C_{DIC}$ was identical between treatments and remained constant during incubation, the higher ε_p during growth under continuous light found by Leboulanger et al. (1995) is consistent with our results for the 4 diatom species belonging to Group I. As in our study, correction for the dark period yields higher μ_i compared to continuous light, and the decrease in ε_p thus correlates with an increase in μ_i.

Effect of CO_2 in relation to growth rate

Skeletonema costatum and *Thalassiosira weissflogii* were used in experiments to test the effect of CO_2 concentration on isotopic fractionation in Group I and

Group II, respectively. Since a decrease in $[CO_{2,aq}]$ from 26 to 3 µmol kg^{-1} had only a small effect on growth rate in both *S. costatum* and *T. weissflogii* (Table 1), the effect of $[CO_{2,aq}]$ on ε_p at constant μ_i could be directly compared to the effect of μ_i on ε_p at constant $[CO_{2,aq}]$. In both species, ε_p decreased by <3‰ from high to low $[CO_{2,aq}]$. This change in ε_p is small compared to the >10‰ latitudinal variation in ε_p of plankton in the ocean, attributed by Rau et al. (1989) to CO_2 concentrations ranging from ca 5 to 25 µmol kg^{-1}. In contrast, an increase in μ_i in response to a shorter daily photoperiod caused a decrease in ε_p by ca 6‰ in *S. costatum*, indicating that daylength-related growth rate effects on isotopic fractionation can be greater than CO_2 effects. If the dominant phytoplankton species in the ocean show a response of ε_p to growth rate similar to that of the Group I organisms in our study, the difference in μ_i between lower latitudes (short days, high temperature) and higher latitudes (long days, low temperature) may contribute significantly to the observed latitudinal variation in isotopic composition of natural plankton assemblages in the ocean. The potential importance of phytoplankton growth rate in contributing to field variability of isotopic fractionation has previously been acknowledged in several other studies (e.g. Francois et al. 1993, Goericke & Fry 1994, Laws et al. 1995, 1997).

Consequences for the ε_p versus μ_i $[CO_{2,aq}]$ relationship

According to existing models of isotopic fractionation in marine phytoplankton (e.g. Francois et al. 1993, Laws et al. 1995, 1997, Rau et al. 1996), both an increase in μ_i and a decrease in $[CO_{2,aq}]$ would lower ε_p. For algal cells of similar carbon content and cell geometry, a linear inverse relationship is predicted between ε_p and $\mu_i/[CO_{2,aq}]$ if inorganic carbon acquisition occurs exclusively by diffusive uptake of CO_2. Even if CO_2 is taken up by an active transport process in addition to passive diffusion, ε_p varies as a linear function of $\mu_i/[CO_{2,aq}]$ as long as the active CO_2 uptake is proportional to intracellular carbon demand or growth rate (Popp et al. 1998). Another factor which may cause a progressive decrease in ε_p with decreasing $[CO_{2,aq}]$ is the gradual induction of active HCO_3^- uptake towards lower CO_2 concentrations. In fact, as argued by Keller & Morel (1999), the observed ε_p versus $\mu_i/[CO_{2,aq}]$ correlation does not permit differentiation between active uptake of CO_2 and of HCO_3^-.

In our study, daylength-dependent fractionation in Group I is consistent with the predicted increase in ε_p upon a decrease in μ_i at constant $[CO_{2,aq}]$. Both passive diffusion of CO_2 and/or active uptake of inorganic carbon may have accounted for the observed trends. On the other hand, constant fractionation independent of daylength-related changes in μ_i indicates that diffusive CO_2 uptake may not be the dominant pathway of C acquisition in Group II species even at the high CO_2 concentrations in our experiments. Constant fractionation at variable μ_i is expected if changes in the uptake rate of CO_2 or HCO_3^- are proportional to changes in carbon fixation rate, mediated by regulated active carbon transport.

The species-specific response to different irradiance cycles has 2 important implications for the interpretation of isotope data. First, if a significant portion of the phytoplankton species in the field adjusts its mode of inorganic carbon uptake in a way that large variations in μ_i are not reflected in isotopic fractionation, an ε_p versus $\mu_i/[CO_{2,aq}]$ relationship is not applicable for the determination of growth rate or CO_2 concentration from isotope data. Second, the results for *Phaeodactylum tricornutum* indicate that even in a single algal species the dependence of ε_p on the growth rate may differ, contingent upon the factor controlling μ_i. While *P. tricornutum* was insensitive to daylength-related changes in μ_i in our study, lower growth rates enforced by N-limited conditions can result in significantly higher fractionation in this species at comparable $[CO_{2,aq}]$ (Laws et al. 1995).

Based on theoretical consideration, cell surface area and either cellular carbon content or cell volume affect isotopic fractionation (e.g. Goericke et al. 1994, Laws et al. 1995, Rau et al. 1996), which has been demonstrated experimentally by Popp et al. (1998). Our results are consistent with their observation that larger organisms fractionate less at comparable growth rates.

Diurnal variation

Carbon metabolism of algal cells is characterized by carboxylation and decarboxylation reactions, catalyzed and regulated by specific enzymes. Most of these reactions involve isotopic fractionation of up to 40‰ (O'Leary et al. 1992, Goericke et al. 1994) and may thus affect the isotopic composition of algal cells. In marine diatoms, inorganic carbon is fixed predominantly by RUBISCO (Mortain-Bertrand et al. 1987a, b, 1988a, b, Descolas-Gros & Oriol 1992). Next to photosynthetic C fixation by RUBISCO, the most important carboxylation reaction is fixation of inorganic carbon by β-carboxylating enzymes, typically PEP-carboxylase (PEPC) or PEP-carboxykinase (PEPCK) (Raven 1997). Since both enzymes use phosphoenol pyruvate (PEP) as a substrate, which is derived from photosynthesis (Calvin cycle), β-carboxylation is ultimately coupled to the presence of light and represents no net fixation of inorganic carbon. Batch culture experiments with

Skeletonema costatum show that the ratio of β-carboxylation to RUBISCO activity remains very low (<1%) in the early exponential growth phase (Descolas-Gros & Fontugne 1985, Descolas-Gros & Oriol 1992). We thus consider β-carboxylation unlikely to account for the diurnal variability in $\delta^{13}C_{POC}$ observed in our study.

Decarboxylation reactions are central to both catabolic pathways (mobilization of energy) and anabolic pathways (biosynthesis). In darkness, when RUBISCO is inactive and no net fixation of inorganic carbon occurs, carbohydrate reserves are used to fuel metabolic reactions and to provide the carbon skeleton for major biochemical compounds such as proteins and lipids. The effect of decarboxylation reactions on $\delta^{13}C_{POC}$ should therefore be most pronounced during the dark period, when carbon fluxes are dominated by respiratory activity. Isotopic fractionation associated with decarboxylation can range from 9 to 32‰ (for reviews see Goericke et al. 1994, Raven 1997) but will affect $\delta^{13}C_{POC}$ only if respired CO_2 is not efficiently refixed in other cellular substrates. Furthermore, $\delta^{13}C_{POC}$ is not affected when carbon compounds of similar isotopic composition as POC are completely respired to CO_2 in catabolic processes, in which case $\delta^{13}C_{CO_2}$ would not differ from $\delta^{13}C$ of the substrate. On the other hand, complete degradation of compounds that deviate from the average isotopic composition of algal carbon may lead to changes in $\delta^{13}C_{POC}$, even when no fractionation occurs during decarboxylation.

In contrast to growth under continuous light, alternating L/D periods lead to partial synchronization of cell division in phytoplankton cells (e.g. Nelson & Brand 1979, Chisholm 1981, Harding et al. 1981, Prézelin 1992). The timing of maximum cell division seems to depend on the respective growth conditions. For example, *Skeletonema costatum* exhibited maximum division rates during the light period in some studies (Jørgensen 1966, Cosper 1982) but during the dark period in others (Eppley et al. 1971). In our experiments, successive cell counts indicated that the majority of cell divisions occurred during the dark period (data not shown). We suggest that the small but significant variation in $\delta^{13}C_{POC}$ during the photoperiod is mainly driven by variable rates of carbon fixation. If synchronized cell division occurs near the end of the dark period, it is likely to be followed by a period of somewhat lower metabolic activity. In experiments with *S. costatum*, Burkhardt et al. (1999) monitored the diurnal variability of the C:N ratio. A pronounced increase in C:N, reflecting accumulation of carbohydrates in the light, may be taken as an indication for high rates of carbon assimilation. Such an increase in C:N was observed in *S. costatum* only in the second half of a 16 h photoperiod, coinciding with an increase in $\delta^{13}C_{POC}$. These observations are consistent with the predicted decrease in fractionation upon an increase in the rate of carbon fixation.

The drop in $\delta^{13}C_{POC}$ during the last hours of darkness, proceeding into the first half of the photoperiod, may be associated with a major metabolic event such as synchronized cell division. Fractionation associated with carboxylation/decarboxylation reactions, complete decarboxylation of ^{13}C-rich organic compounds, or the loss of ^{13}C-enriched dissolved organic carbon from the cell would lead to ^{12}C enrichment of POC in the dark. The available information, however, prevents identification of causes for the observed trend. While metabolic processes such as respiration and biosynthesis may involve large fractionation by the respective enzymes, the overall effect on $\delta^{13}C_{POC}$ appears to be small, as indicated by the daily amplitude of algal isotopic composition of ≤1.5‰.

CONCLUSIONS

Incubation of phytoplankton at different L/D cycles provides the means to test the effect of growth rate on isotopic fractionation independent of limitation by light intensity or nutrient supply. Laboratory experiments with 7 marine phytoplankton species revealed that duration of the daily photoperiod had a significant impact on ε_p in 4 of the species tested. We attribute this variation in ε_p to the ability of the cells to accelerate the rate of photosynthetic carbon fixation during growth at shorter daylengths. Such a physiological adaptation may be mediated by an increase in cellular activity of RUBISCO. In spite of daylength-related differences in μ_i, no effect of daylength on isotopic fractionation was found in 3 of the species tested, indicating that at least 2 groups of marine microalgae exist which respond differently to variable irradiance cycles. Regulated active uptake of inorganic carbon may be responsible for the observed pattern.

Our data provide evidence that μ_i-dependent fractionation is both species-specific and dependent on the factor controlling growth rate. Thus, a general relationship between ε_p and $\mu_i/[CO_{2,aq}]$ may not exist. These results suggest that *in situ* growth rates of phytoplankton cannot be estimated from a ε_p versus $\mu_i/[CO_{2,aq}]$ relationship based on bulk samples of POC, even if other parameters such as cell size and cell geometry are well constrained. It needs to be investigated whether the use of species-specific or group-specific biomarkers, as for example alkenones of coccolithophorids, can sufficiently reduce the complexity of interpreting isotope data in paleo-reconstruction, as has been suggested in previous studies (e.g. Jasper & Hayes 1990, 1994, Francois et al. 1993, Goericke & Fry 1994).

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